



TITLE:

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**TLR2-MyD88-dependent PI3K and Rac1 activation facilitates the
phagocytosis of *Listeria monocytogenes* by murine macrophages**

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Abstract

Toll-like receptors (TLRs) play a key role in the innate immune response by sensing bacterial ligands. The mechanisms involved in the TLR-mediated cytokine response are well established, however, the possible contribution of TLR-dependent recognition of bacteria to macrophage phagocytosis remains unclear. *Listeria monocytogenes* (LM) is an intracellular parasitic Gram-positive bacterium recognized mainly by TLR2. In this study, we investigated whether TLR2-dependent signaling is involved in the phagocytosis of LM by macrophages. We found no difference in the number of LM associating with wild type (WT) and TLR2^{-/-} macrophages 1 h after infection. However, the number of LM phagocytosed in TLR2^{-/-} and MyD88^{-/-} macrophages was significantly lower compared with WT macrophages. In addition, LPS treatment restored impaired phagocytic activity of TLR2^{-/-} macrophages but did not enhance the activity of MyD88^{-/-} macrophages. The efficiency of phagocytosis was suppressed by inhibitors of phosphatidylinositol 3-kinase (PI3K) and the small Rho GTPases, but not by cycloheximide. Moreover, functional activation of PI3K and Rac1 was impaired in TLR2^{-/-} and MyD88^{-/-} macrophages. In an *in vivo* infection model, we found significantly lower numbers of LM phagocytosed in peritoneal macrophages of TLR2^{-/-} and MyD88^{-/-} mice after intraperitoneal infection. Moreover, a lower number of bacteria were detected in the spleen of TLR2^{-/-} mice 1 day after intravenous infection compared with WT mice. These results clearly indicated that TLR2-MyD88-dependent signaling enhances the basal level of phagocytosis of LM by macrophages through activation of PI3K and Rac1, not by synthesis of proinflammatory cytokines or expression of phagocytic receptors.

1 Introduction

2 *Listeria monocytogenes* (LM) is a Gram-positive facultative intracellular bacterium that
3 causes severe disease (listeriosis) in humans and various animal species with a mortality
4 rate of approximately 30% (14, 38). LM can invade various types of cells including
5 epithelial cells, hepatocytes, endothelial cells, fibroblasts and macrophages. After entry
6 into host cells, LM is trapped temporarily in an endosome. However, the bacterium
7 quickly escapes from the endosome into the cell cytoplasm where it undergoes rapid
8 replication by means of expressing various virulence genes including *prfA*, *plcA*, *hlyA*,
9 *actA*, *mpl* and *plcB*, all of which are located in a locus called *Listeria* pathogenicity
10 island 1 (14).

11 Accumulating evidence suggests that LM gains entry into nonprofessional phagocytes
12 by utilizing bacterial invasion factors called internalins (11, 20, 24, 34, 42, 46). Indeed,
13 internalin A mediates the entry of LM into human intestinal cells through binding to E-
14 cadherin and the interaction of internalin B with c-Met (hepatocyte growth factor
15 receptor) induces endocytosis of the bacterium into various types of cells (8, 25, 37). In
16 comparison, professional phagocytes such as macrophages are able to phagocytose and
17 subsequently kill various pathogens through phagolysosome fusion. Macrophages are
18 known to express various receptors that recognize bacterial components or bind to
19 opsonins attached to bacteria (54), a series of intracellular signaling pathways are then
20 activated that lead to the dynamic and rapid reorganization of the actin cytoskeleton for
21 phagocytic engulfment. Of these receptors, Fcγ receptor and complement receptor serve
22 as opsonin receptors for IgG and C3b, respectively, that bind to the surface of bacteria
23 (19, 33). Mannose receptor and CD14 can contribute to phagocytosis of bacteria
24 through direct binding to mannosylated components (15, 17, 45). Scavenger receptor is

also implicated in phagocytosis (43). These different cell surface receptors likely operate alone or in combination in the recognition and efficient internalization of bacteria into macrophages.

Toll-like receptors (TLRs) are type I integral membrane glycoproteins that are expressed in all lymphoid tissues with the highest expression in peripheral blood leukocytes. TLRs are pattern recognition receptors (PRR) characterized by the presence of a Toll/IL-1 receptor (TIR) domain homologous to interleukin-1 receptor (IL-1R) in their cytoplasmic portion and a variable number of leucine-rich repeats (LRR) in their extracellular portion (31). There are more than ten TLRs identified to date in mammals, and each TLR exhibits a distinct function in innate immune recognition (1, 5, 52). For example, TLR2 recognizes lipoteichoic acid, peptidoglycan and lipoproteins of Gram-positive bacteria. TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria and TLR9 recognizes bacterial hypomethylated CpG DNA motifs. TLR-dependent recognition of bacteria induces a signal through myeloid differentiation factor 88 (MyD88) and is accompanied by inflammatory responses in macrophages (51). TLR2-MyD88 signaling is known to be critical for the pro-inflammatory cytokine response during LM infection. Several studies *in vivo* have revealed that TLR2 is required for optimum control of LM infection (29, 53). The increased susceptibility of MyD88^{-/-} mice to LM infection may support the idea that TLR2 plays a role in resistance to LM (47). On the other hand, Edelson et al. showed that MyD88 was necessary for resistance to LM infection but TLR2 deficiency did not influence the propagation of LM *in vivo* (16). These conflicting findings appeared to indicate that though TLR2 participates in host resistance to LM through the induction of cytokine

production *in vivo*, there are some signaling pathways that compensate the lack of TLR2 to control bacterial infection.

In addition to this established function of TLRs in the host cytokine response, recent studies suggested that TLR signaling modulates the phagocytosis of pathogens (27, 41). Indeed, Blander and Medzhitov (6) reported that TLR-mediated signaling regulates phagolysosomal maturation in bone-marrow-derived macrophages after infection with *Escherichia coli*, heat-killed *Salmonella* Typhimurium and *Staphylococcus aureus*. Letiembre *et al.* (35) reported that TLR2 promotes the phagocytosis of *Streptococcus pneumoniae* and killing of bacteria by polymorphonuclear leukocytes. Moreover, Luther *et al.* (39) have shown that TLR2, MyD88 and dectin-1 are required for efficient phagocytosis of *Aspergillus fumigatus* conidia. Doyle *et al.* (13) also reported that TLR-mediated enhancement of phagocytosis is due to the up-regulation of scavenger receptors, and another recent report on MyD88-mediated phagocytosis of *Borrelia burgdoferi* has emphasized the importance of downstream signaling through PI3K (48). From these findings it is likely that TLR signaling contributes to the phagocytosis and phagolysosome formation in professional phagocytes upon infection with extracellular parasitic bacteria or killed bacteria. On the other hand, little is known about whether the phagocytosis of LM, a representative Gram-positive intracellular parasitic bacterium, is dependent on the TLR signaling pathway. In this study, we analyze the contribution of TLR2-MyD88 signaling to phagocytosis of LM both *in vitro* and *in vivo*.

Materials and Methods

Reagents

Lipopolysaccharide (LPS) and *Clostridium difficile* toxin B, a small Rho GTPase inhibitor, were purchased from Sigma-Aldrich (Tokyo, Japan). Pam3CSK4 was purchased from Invivogen (San Diego, CA, USA). LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, was obtained from Calbiochem (Tokyo, Japan). Gentamicin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Tryptic soy agar and thioglycollate medium were purchased from EIKEN Chemical (Tokyo, Japan). 5- or 6-(*N*-succinimidylloxycarbonyl)-fluorescein 3', 6'-diacetate (CFSE) and Bacstain CTC (5-cyano-2, 3-ditolyl-2*H*-tetrazolium chloride) Rapid Staining kit were purchased from DOJINDO (Kumamoto, Japan). Cycloheximide (CHX) and a protease inhibitor cocktail were purchased from Nacalai Tesque (Kyoto, Japan). Polystyrene microspheres (1 μ m in diameter) were obtained from Polysciences (Warrington, PA). EZ-Detect™ Rac1 activation kit and EZ-Detect™ Cdc42 activation kit were purchased from Pierce Chemical (Rockford, IL). Recombinant mouse M-CSF was purchased from R&D Systems (Minneapolis, MN).

Antibodies

Alexa Fluor 594-conjugated anti-rabbit IgG antibody (Ab) and Alexa Fluor 350-conjugated anti-rabbit IgG Ab were purchased from Invivogen (San Diego, CA, USA). Anti-TLR2 monoclonal Ab (mAb, T2.5, mouse IgG1), FITC-conjugated anti-mouse TLR2 mAb (6C2, rat IgG2b), PE-conjugated anti-F4/80 mAb (BM8, rat IgG2a) and isotype-matched control mAbs (rat IgG2b and rat IgG2a) were obtained from eBioscience (San Diego, CA, USA). Affinity-purified mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and used as a control IgG for anti-TLR2 mAb (T2.5). Rabbit anti-*Listeria* polyclonal Ab (IgG) was purchased from

VivoStat (Alleroed, Denmark). Rabbit anti-Akt polyclonal Ab (IgG) and rabbit anti-phosphorylated Akt mAb (IgG2b) were purchased from Cell Signaling Technology (Tokyo Japan). Anti-Rac1 mAb (mouse IgG2b) and anti-Cdc42 mAb (mouse IgG1) were obtained from Pierce (Rockford, IL, USA). Rat anti-mouse CD16/CD32 mAb (2.4G2, rat IgG2b) which specifically recognizes mouse FcγIII (CD16) and FcγII (CD32) receptors was obtained from BD Biosciences (Tokyo, Japan).

Animals used in this study

Wild-type female C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). TLR2^{-/-}, TLR4^{-/-} and MyD88^{-/-} mice on a C57BL/6 background were purchased from Oriental Bioservice (Kyoto, Japan). All mice were maintained in specific-pathogen-free conditions and used at 7 to 10 weeks of age. The Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine (Kyoto, Japan) approved all of the experimental procedures performed.

Bacterial strains used in this study

Listeria monocytogenes (LM) strain EGD was grown overnight in brain heart infusion (BHI) broth (EIKEN Chemical, Tokyo, Japan) at 37°C with shaking. One volume of the bacterial suspension was added to one hundred volumes of fresh BHI broth and LM was cultured for 5 h. After being washed, bacteria were suspended in phosphate-buffered saline (PBS) supplemented with 10% glycerol and stored at -80°C in aliquots. The concentration of bacteria was determined by plating 10-fold serially diluted suspensions on tryptic soy agar plates and counting the number of colonies 24 h after incubation.

Enumeration of associating LM and LM phagocytosed by macrophages

C57BL/6 WT, TLR2^{-/-}, TLR4^{-/-} and MyD88^{-/-} mice were injected intraperitoneally with 3 ml of 3% thioglycollate medium. Peritoneal exudate cells (PECs) were collected four days later. Bone marrow cells were collected from tibiae of mice and cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 µg ml⁻¹ gentamicin and recombinant mouse M-CSF (100ng ml⁻¹) for 7 days. After washing with RPMI1640 medium, adherent bone marrow-derived macrophages were collected. Cells were seeded in a 48-well plate at 2.5 × 10⁵ cells well⁻¹ and incubated at 37°C for 2 h in RPMI1640 medium supplemented with 10% FBS. After washing to remove nonadherent cells, adherent macrophages were infected with LM at a multiplicity of infection (MOI) of 20 at 37°C for 1 h in FBS-free RPMI1640 medium. To enumerate LM associating with macrophages, cells were washed thoroughly to remove non-associating bacteria and lysed in PBS containing 0.1% Triton X-100. The cell lysate was diluted with PBS and inoculated on tryptic soy agar plates. The colony forming unit (CFU) number was counted 24 h after incubation. To enumerate the internalized bacteria, macrophages were infected with LM as described above, washed and cultured for 30 min in the presence of 20 µg ml⁻¹ gentamicin. Preliminary experiment showed that extracellular bacteria are completely killed by this antibiotic treatment. Cells were washed and lysed in PBS containing 0.1% Triton X-100. The CFU number in the cell lysate was determined. In some experiments, macrophages were treated with 40 µg ml⁻¹ anti-TLR2 mAb, 20 µM LY294002, 100 µg ml⁻¹ CHX or 40 ng ml⁻¹ *C. difficile* toxin B for 1 h, and infected with LM. Alternatively, WT, TLR2^{-/-} and MyD88^{-/-} macrophages were infected with LM at a MOI of 20 for 1 h in the presence of 100 ng ml⁻¹ LPS (TLR4 ligand) or 100 ng ml⁻¹ Pam3CSK4 (TLR2 ligand) and the

number of internalized LM was evaluated. Results were expressed as the relative phagocytic index which was calculated using the following formula: phagocytic index = (the number of phagocytosed LM / the number of associating LM) \times 100.

Discrimination between adherent and phagocytosed LM by immunofluorescence microscopy

LM was labeled with 1 $\mu\text{g ml}^{-1}$ CFSE for 15 min at room temperature with gentle shaking in the dark. Bacteria were washed twice with PBS and suspended in RPMI 1640 medium. PECs obtained from WT, TLR2^{-/-} and MyD88^{-/-} mice were seeded at 2×10^5 cells well⁻¹ in a 24-well plate in which a 12 mm glass cover slip was placed in each well and washed 2 h after incubation to remove nonadherent cells. Adherent macrophages were infected with CFSE-labeled LM for 1 h, washed and fixed with PBS containing 1% paraformaldehyde for 5 min at room temperature. After confirming that the phagocytic efficiency was almost constant when cells were infected at a MOI of 20 to 50, we infected macrophages with LM at MOI of 50 as this MOI enabled us to easily distinguish the associating bacteria from phagocytosed LM. To distinguish phagocytosed LM from adherent LM, infected cells were treated with rabbit anti-*Listeria* Ab and Alexa Fluor 594-conjugated anti-rabbit IgG Ab to stain only the extracellular LM as described previously (22). The number of associating LM and adherent LM to macrophages were counted under a fluorescent microscope. Phagocytosed LM was enumerated using the following equation: the number of phagocytosed LM = the number of associating LM – the number of adherent LM. In addition, macrophages were incubated with polystyrene microspheres for 2 h at a bead to cell ratio of 50 to 1, and the phagocytic activity was evaluated.

1

2 *Viability of phagocytosed LM in macrophages*

3 Based on our preliminary finding that only the viable LM is able to convert CTC into
4 fluorescent insoluble formazan, we employed the CTC staining kit to distinguish viable
5 cells of LM from dead LM inside macrophages. To estimate the viability of
6 phagocytosed LM in macrophages, therefore, PECs from WT, TLR2^{-/-} and MyD88^{-/-}
7 mice were infected with CFSE-labeled LM at a MOI of 50 for 1 h. After washing, cells
8 were incubated with CTC for 15 min to stain LM associating with macrophages. Cells
9 were washed and fixed in PBS containing 1% paraformaldehyde. The extracellular LM
10 was then stained with rabbit anti-*Listeria* Ab and Alexa Fluor 350-conjugated anti-
11 rabbit IgG Ab. The number of viable and dead bacteria phagocytosed in macrophages
12 was counted under a fluorescence microscope. The viability of LM (%) was calculated
13 as follows: the number of viable LM/ total number of phagocytosed LM × 100.

14

15 *Flow cytometric analysis*

16 Peritoneal exudate macrophages (1×10^6 cells) were prepared and treated with anti-
17 mouse CD16/CD32 mAb to block nonspecific binding to Fc receptor. In some
18 experiment, peritoneal exudate macrophages were incubated for 1 h with 20 μ M
19 LY294002 or 40 ng ml⁻¹ toxin B before treatment with anti-mouse CD16/CD32 mAb.
20 Cells were stained with anti-TLR2 mAb (6C2) at a concentration of 0.5 μ g ml⁻¹ for 30
21 min on ice and analyzed on a FACScalibur flow cytometer using the CELL Quest
22 software (Becton Dickinson, Tokyo, Japan) (18). To determine the percentage of
23 macrophages in PECs of WT, TLR2^{-/-} and MyD88^{-/-} mice, PECs were collected, stained
24 with PE-conjugated anti-F4/80 mAb and analyzed by flow cytometry.

Determination of phosphatidylinositol 3-kinase (PI3K) activity by detection of phosphorylated Akt

Akt is a downstream substrate of PI3K and phosphorylation of Akt can be used as a marker of PI3K activation (2). Thus, we analyzed the amount of phosphorylated Akt semi-quantitatively by Western blotting. Peritoneal exudate macrophages were prepared at 1×10^6 cells well⁻¹ in a 24-well culture plate and infected with LM (MOI=20) for 15 and 30 min. The cells were lysed in 100 μ l of 2 \times SDS sample buffer containing a protease inhibitor cocktail. The cell lysate was sonicated for 5 min, boiled for 2 min and then subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins were electroblotted to PVDF membrane. The membrane was incubated with rabbit anti-Akt Ab or rabbit anti-phosphorylated Akt mAb for 1 h, and anti-rabbit IgG Ab conjugated with horseradish peroxidase. After treatment with Enhanced Chemiluminescence (ECL) Detection Reagents (Millipore, Billerica, MA, USA), the bands representative of Akt and phosphorylated Akt were detected using LAS-4000 mini Fuji film (Tokyo, Japan).

Determination of Rac1 and Cdc42 activation in LM-infected macrophages

Activated Rac1 and Cdc42 were detected using an EZ-DetectTM Rac1 and Cdc42 activation kits. Briefly, peritoneal exudate macrophages were prepared at 2×10^6 cells well⁻¹ in a 24-well culture plate and infected with LM (MOI=20) for 15 and 30 min. The cells were lysed in a lysis/binding/wash buffer (Pierce) containing a protease inhibitor cocktail and the supernatant was collected after centrifugation ($16,000 \times g$) at 4°C for 15 min. Activated Rac1 and Cdc42 were precipitated with GST-human Pak1-PBD and detected with mouse anti-Rac1 mAb and anti-Cdc42 mAb by Western blotting,

respectively.

Phagocytosis of LM by macrophages during in vivo infection

WT, TLR2^{-/-} and MyD88^{-/-} mice were injected intraperitoneally (i.p.) with 3 ml of 3% thioglycollate medium and infected i.p. with 1×10^8 CFU of LM 4 days later. PECs were harvested 5 min after infection, washed and cultured in RPMI1640 medium containing gentamicin for 30 min to kill extracellular LM. Cells were washed and lysed by 0.1% Triton X-100. The number of phagocytosed LM was determined as described above. Alternatively, WT and TLR2^{-/-} mice were injected intravenously (i.v.) with 10^4 CFU of LM. Spleen was collected 1 day after infection and homogenized in PBS. The number of viable bacteria in the homogenates was determined.

Statistical analysis

The statistical significance of the data was determined using a two-tailed Student's *t*-test. A *P* value of < 0.05 was considered statistically significant. All data represent the results of two to three independent experiments.

Results

TLR2, but not TLR4, is involved in the phagocytosis of LM by macrophages

To analyze the role of TLR2 in the phagocytosis of LM, we infected macrophages with LM (5×10^6 CFU) in the presence or absence of anti-TLR2 mAb and determined the number of internalized bacteria using the gentamicin protection assay. As shown in Figure 1A, WT macrophages were able to phagocytose about 5×10^5 CFU of LM, which was equivalent to 10% of the total bacteria added to the culture. Treatment with

1 anti-TLR2 mAb significantly reduced the number of internalized bacteria while mouse
2 IgG, used as a control, did not affect the ability of macrophages to phagocytose bacteria
3 (Fig. 1A). To confirm the involvement of TLR2 in the phagocytosis of LM,
4 macrophages from WT, TLR2^{-/-} and TLR4-deficient (TLR4^{-/-}) mice were compared. As
5 expected, the phagocytic ability of TLR2^{-/-} macrophages was significantly lower than
6 that of WT macrophages (Fig. 1B). On the other hand, TLR4^{-/-} macrophages did not
7 show any defect in phagocytic ability compared with WT macrophages. As TLR2 has
8 been shown to recognize components of LM (39), it is postulated that TLR2 serves as a
9 phagocytic receptor that traps LM directly on macrophages. Alternatively, the
10 intracellular signaling that is triggered by recognition of LM by TLR2 may be critical
11 for the phagocytosis of LM. To investigate this, we infected WT and TLR2^{-/-}
12 macrophages with LM and determined the number of both LM associating with
13 macrophages and phagocytosed bacteria. There was no difference in the number of
14 associating LM between WT and TLR2^{-/-} macrophages, indicating that the bacterium
15 adhered to macrophages irrespective of the expression of TLR2 (Fig. 1C). On the other
16 hand, WT macrophages phagocytosed about 55 % of LM out of all associating bacteria;
17 and TLR2^{-/-} macrophages exhibited a significantly lower phagocytic index (Fig. 1D).
18 When bone marrow-derived macrophages (BMDM) were used, a similar difference was
19 observed between WT BMDM and TLR2^{-/-} BMDM (Fig. 1E). To rule out the
20 possibility of a general impairment in phagocytic ability, we analyzed the internalization
21 of polystyrene beads, which is not dependent on any TLRs. As shown in Figure 1F, the
22 internalization of polystyrene beads was found to be identical in WT and TLR2^{-/-}
23 macrophages. These results showed that TLR2, but not TLR4, might play a role in the
24 phagocytosis of LM by macrophages.

1
2 To further confirm the involvement of TLR2 in the phagocytosis of LM, the phagocytic
3 efficiency was assessed by fluorescence microscopy. We infected WT and TLR2^{-/-}
4 macrophages with CFSE-labeled LM. Cells were then treated with rabbit anti-*Listeria*
5 Ab and Alexa Fluor 594-conjugated anti-rabbit IgG Ab. This procedure enabled us to
6 distinguish internalized bacteria from bacteria adhering to the surface of macrophages
7 under fluorescence microscope (Fig. 2A). The efficiency of phagocytosis was
8 determined in 500 infected macrophages. As being similar to Fig. 1C, there was no
9 difference in the total number of associating LM between WT and TLR2^{-/-} macrophages
10 (Fig. 2B). On the other hand, WT macrophages exhibited a higher phagocytic activity
11 than TLR2^{-/-} macrophages. The phagocytic index was found to be significantly lower in
12 TLR2^{-/-} macrophages compared to that of WT (Fig. 2C). We further compared the
13 phagocytic efficiency of individual macrophages between the two groups. The results
14 depicted in Figure 2D illustrating the number of macrophages grouped according to
15 their phagocytic index in WT and TLR2^{-/-} macrophages, clearly shows that most of the
16 WT macrophages had a higher phagocytic index whereas most of the TLR2^{-/-}
17 macrophages had a lower phagocytic index. Furthermore, it is probable that the
18 difference in the phagocytic efficiency between WT and TLR2^{-/-} macrophages might be
19 attributable to the bactericidal activity of these macrophages. To test the hypothesis, the
20 viability of LM inside macrophages was measured after 1 h of phagocytosis. We found
21 that most of the bacteria inside WT and TLR2^{-/-} macrophages were viable and there was
22 no significant difference in the viability between these two groups (Fig. 2E). These
23 results indicated that TLR2 participates in the enhancement of the basic phagocytosis of
24 LM by macrophages while TLR2 itself does not participate in the direct adhesion of LM.

MyD88 is required for efficient TLR2-dependent phagocytosis of LM

We next investigated whether TLR2-dependent signaling is required for phagocytosis of LM. As MyD88 is an essential adaptor protein for TLR2-dependent signaling, we examined the phagocytic ability of macrophages from MyD88^{-/-} mice. As shown in Figure 3A, the number of phagocytosed LM in MyD88^{-/-} macrophages was significantly lower than that in WT macrophages. In addition, fluorescence microscopy revealed that MyD88^{-/-} macrophages had a significantly lower phagocytic index compared with WT macrophages (Fig. 3B). The absence of any reduction in the phagocytosis of polystyrene beads supported that the view that the effect seen in MyD88^{-/-} macrophages was not due to some general impairment of macrophage function (Fig. 3C). In addition, we found that there was no difference in the viability of bacteria in WT and MyD88^{-/-} macrophages. The majority of phagocytosed bacteria were viable in two groups of macrophages (Fig. 3D). These results indicated that TLR2- and MyD88-dependent signaling enhanced the phagocytosis of LM by macrophages. To rule out the possibility that the reduced phagocytosis of LM observed in MyD88^{-/-} macrophages is due to the reduced expression of TLR2, we carried out FACS analysis to analyze the expression level of TLR2 on these macrophages. As shown in Figure 3E, TLR2 was expressed on WT but not TLR2^{-/-} macrophages. In MyD88^{-/-} macrophages, the expression level of TLR2 was slightly reduced but a substantial expression was detected. In addition, we determined the percentage of macrophages in whole and adherent PECs of WT, TLR2^{-/-} and MyD88^{-/-} mice by measuring the number of F4/80-positive cells. There was no difference in the purity of macrophages in whole and adherent PECs of these mice (Fig. 3F). From these findings, it was clear that the

reduced phagocytic efficiency in MyD88^{-/-} macrophages was not due to the reduced expression of the extracellular domain of TLR2, a general impairment in phagocytosis or a difference in bactericidal activity. It was also clear that the TLR-MyD88 signaling pathway plays a potentially important role in intracellular engulfment of bacteria adhering to the macrophage surface.

Involvement of downstream signaling from TLR-MyD88 but not cytokine/other gene expression in phagocytosis of LM by macrophages

LPS is known to stimulate the cytokine response through TLR4-MyD88-dependent signaling pathway. To confirm the importance of the MyD88-dependent signaling pathway, we tested whether stimulation with LPS restores the level of LM phagocytosis in TLR2^{-/-} or MyD88^{-/-} macrophages to WT levels. WT, TLR2^{-/-} and MyD88^{-/-} macrophages were infected with LM in the presence or absence of LPS or Pam3CSK4 (an authentic TLR2 ligand), and the efficiency of phagocytosis was examined. The phagocytic ability of WT macrophages was not influenced by treatment with either Pam3CSK4 or LPS. Again, TLR2^{-/-} macrophages exhibited a decrease in the phagocytic index compared with WT macrophages, as shown previously. However, the reduced phagocytic ability of TLR2^{-/-} macrophages was significantly increased by treatment with LPS, while Pam3CSK4 did not show such an enhancing effect (Fig. 4A). On the other hand, treatment with LPS never enhanced the phagocytic efficiency of MyD88^{-/-} macrophages (Fig. 4B). These results strongly suggested that activation of the MyD88-dependent signaling pathway is required for the enhancement of basic phagocytosis of LM by macrophages.

It has been shown that activation of the TLR2 signaling pathway induces the production of cytokines, including tumor necrosis factor- α (TNF- α) that causes macrophage activation. Doyle *et al.* (13) recently reported that stimulation of macrophages with TLR ligands induces the expression of several scavenger receptors that serve as phagocytic receptors. Therefore, it could be postulated that signals downstream of the TLR-MyD88 pathway, leading to expression of cytokines or some phagocytic receptors after LM infection, might contribute to phagocytosis. To test this, we determined the effect of CHX on phagocytosis. CHX treatment mostly inhibited the production of IL-6 and TNF- α from infected macrophages, whereas cell viability was not affected (data not shown). We found that CHX treatment did not alter the number of phagocytosed bacteria by WT and TLR2^{-/-} macrophages (Fig. 4C), suggesting that the phagocytosis of LM is independent of protein synthesis and that TLR2-dependent enhancement of phagocytosis is not due to cytokine production or expression of phagocytic receptors.

Phosphatidylinositol 3-kinase (PI3K) is implicated in TLR2-dependent enhancement of phagocytosis of LM

Phagocytosis of bacteria requires the activation of a number of signaling pathways that regulate the rearrangement of the actin cytoskeleton and extension of the plasma membrane (54). It has been shown that PI3K plays an important role in phagocytosis and TLR-dependent signaling induces activation of PI3K (32). These findings prompted us to determine whether PI3K contributes to the TLR2-dependent phagocytosis of LM. We infected WT, TLR2^{-/-} and MyD88^{-/-} macrophages with LM in the presence or absence of LY294002, a PI3K inhibitor, and measured the number of phagocytosed bacteria. In WT macrophages, PI3K inhibitor reduced the efficiency of phagocytosis in

a dose dependent manner (Fig. 5A). We found that treatment of WT macrophages with LY294002 decreased the number of phagocytosed bacteria to almost the basal level observed in TLR2^{-/-} and MyD88^{-/-} macrophages. However, the inhibitor did not affect the phagocytosis by TLR2^{-/-} and MyD88^{-/-} macrophages (Fig. 5B). To confirm whether activation of PI3K is actually induced in infected macrophages, we analyzed the amount of phosphorylated Akt, one of the substrates of PI3K known to be an important signaling molecule for the induction of phagocytosis (2). During infection of WT macrophages with LM, the amount of phosphorylated Akt (pAKT) was increased 30 min after infection. However, no increase in pAKT was detected in TLR2^{-/-} and MyD88^{-/-} macrophages (Fig. 5C). These results indicated that LM-induced TLR2 signaling enhances phagocytosis through activation of PI3K and Akt phosphorylation.

Rac1 but not Cdc42 activation is important for the phagocytosis of LM by macrophages

PI3K signaling has been reported to play an important role in actin polymerization through activation of the Rho family of GTPases that subsequently recruit the Arp2/3 complex to form the actin nucleation (7). In order to determine the possible involvement of these Rho family GTPases in the PI3K-directed actin polymerization during phagocytosis of LM, we tested the effect of *C. difficile* toxin B, an inhibitor specific for the Rho family GTPases (9, 30). We infected WT, TLR2^{-/-} and MyD88^{-/-} macrophages with LM in the presence or absence of toxin B, and measured the number of phagocytosed bacteria. In WT macrophages, toxin B markedly reduced the efficiency of phagocytosis but did not affect phagocytosis by TLR2^{-/-} and MyD88^{-/-} macrophages (Fig. 6A). We also examined whether Rac1 activation is induced in infected macrophages by analyzing the activated GTP-bound form of Rac1 or Cdc42. According

to Western blot analysis (Fig. 6B), an increased level of GTP-bound Rac1 was detected 30 min after infection in WT macrophages whereas it was almost undetectable in TLR2^{-/-} and MyD88^{-/-} macrophages. Cdc42 did not appear to be involved in the process because there was no difference in the levels of GTP-bound Cdc42 between WT macrophages and TLR2^{-/-} or MyD88^{-/-} macrophages. In addition, the expression level of TLR2 on WT, TLR2^{-/-} and MyD88^{-/-} macrophages was not affected by treatment with LY294002 or toxin B (Fig. 7). Taken together, these results indicated that LM-induced TLR2-MyD88 signaling enhances the phagocytosis through activation of PI3K and Rac1.

TLR-MyD88 signaling is important for the phagocytosis during LM infection in vivo

In order to confirm the biological significance of our *in vitro* data during LM infection *in vivo*, we compared the number of phagocytosed bacteria in WT, TLR2^{-/-} and MyD88^{-/-} mice i.p. infected with LM. As shown in Figure 8A, the number of LM entrapped by a given number of macrophages recovered after i.p. infection was significantly lower in TLR2^{-/-} and MyD88^{-/-} mice as compared with the macrophages from WT mice. It has been shown that LM is captured mainly by various types of macrophages in the periarteriolar lymphoid sheath within 24 h after infection (4). Therefore, we intravenously infected WT and TLR2^{-/-} mice with LM and the CFU number in spleen were enumerated 1 day later. The CFU number in spleen of TLR2^{-/-} mice was approximately 4 times as low as that of WT mice (0.5×10^5 CFU Vs 1.9×10^5 CFU, respectively) (Fig. 8B). Thereafter, LM replicated similarly in spleens of both WT and TLR2^{-/-} mice and the CFU number reached at 1×10^7 CFU in WT and 0.8×10^7 CFU in TLR2^{-/-} mice 3 days after infection. These results suggest that the TLR2-

dependent signaling actually contributes to the phagocytosis of macrophages *in vivo* and the phagocytic activity of TLR2^{-/-} macrophages might result in the lower bacterial burden within 1 day after LM infection whereas the difference in the phagocytic activity did not affect the final bacterial growth at the late phase of infection.

Discussion

Increasing evidences indicate that TLR-dependent signaling promotes not only cytokine production but also phagocytosis of bacteria by macrophages. The effect of TLR on phagocytosis of some extracellular bacteria has previously been reported (6, 13, 35). TLR2 expressed on macrophages plays a key role in the cellular response to components of Gram-positive bacteria including LM (28, 36). However, there have been no reports to date showing that TLR2 signaling is involved in the phagocytosis of LM by macrophages. The results obtained in this study have clearly shown that although LM is able to invade macrophages independently of TLR2 and MyD88, TLR2 but not TLR4, enhances the basic level of phagocytosis of LM by macrophages.

It has been shown that intracellular parasitic bacteria are capable of modulating the bactericidal activity of macrophages in order to grow and reside in infected cells. LM is known to survive inside macrophages by escaping from phagosomes into the cytosol before phagolysosome formation takes place. However, Alvarez-Dominguez *et al.* (3) and Henry *et al.* (23) suggested that virulent LM has the ability to delay phagosome maturation. Although it has not yet been elucidated how LM directly manipulates the bactericidal mechanism of professional phagocytes, it is clear that LM has the potential to modulate the intracellular killing mechanism. Therefore, we determined the

1 contribution of TLR2 signaling in the phagocytosis of LM by macrophages. Here we
2 have shown that peritoneal macrophages from TLR2^{-/-} and MyD88^{-/-} have markedly
3 reduced phagocytic efficiency compared with WT macrophages. A similar reduction
4 was observed in bone marrow-derived macrophages from these knockout mice although
5 the number of phagocytosed LM were 5-fold lower than peritoneal macrophages (data
6 not shown). Therefore it is clear that the TLR2-MyD88-dependent signaling pathway is
7 critical for the efficient phagocytosis of LM.

8
9 Doyle *et al.* (13) showed that pretreatment with TLR ligands enhances phagocytosis by
10 macrophages due to up-regulation of specific phagocytic receptors such as scavenger
11 receptors, including scavenger receptor-A (SR-A), a macrophage receptor with a
12 collagenous structure (MARCO). However, in our experiments, the difference in the
13 efficiency of phagocytosis between WT and TLR2^{-/-} macrophages could be observed as
14 early as 1 h after infection. In addition, treatment with CHX did not affect the levels of
15 phagocytosis in WT and TLR2^{-/-} macrophages. Although any level of contribution of
16 known phagocytic receptors that are constitutively expressed cannot be completely
17 excluded, these results strongly imply that TLR2-dependent enhancement of
18 phagocytosis of LM is not simply due to the production of cytokines or expression of
19 phagocytic receptors such as scavenger receptors after LM infection.

20
21 In studying the possible mechanism involved in the TLR-mediated enhanced
22 phagocytosis of LM by macrophages, fluorescence microscopy was useful in
23 distinguishing LM outside or inside the macrophages. From our findings, TLR2 is
24 unlikely acting as a phagocytic receptor that directly captures LM on macrophage

surfaces. Shin *et al.* (48) have shown that signaling through TLR adaptor MyD88 plays an important role in the uptake of *Borrelia*. Our results showing the reduced efficiency of phagocytosis in MyD88^{-/-} macrophages suggested a similar role of TLR-MyD88 signaling in phagocytosis of LM by macrophages. Moreover, the restoration of reduced phagocytosis in TLR2^{-/-} but not MyD88^{-/-} macrophages by addition of the TLR4 ligand LPS confirmed the importance of downstream signaling through MyD88 in the enhancement of phagocytosis.

MyD88 is an adaptor protein involved in a variety of downstream signaling pathways (1, 41), and some reports have suggested the link between PI3 kinases and phagocytosis (49, 54). PI3K catalyzes the phosphorylation of several species of phosphatidylinositol that are important in recruiting signaling molecules such as Akt/PKB to specific regions of the membrane. It is also involved in membrane extension, fusion behind bound particles and insertion of new membrane at the site of particle internalization (54). Recent studies have shown that PI3K is involved in the induction of TLR-mediated cellular responses including uptake of TLR ligands and cytokine production (32, 50). In our study we found that preincubation with LY294002, a PI3K inhibitor, resulted in impaired phagocytosis of LM by WT macrophages in a dose dependent manner. However, treatment with p38 and ERK inhibitors did not result in a reduction in phagocytosis of LM by macrophages (data not shown). Furthermore, there was a significant difference in the magnitude of PI3K activation between WT and TLR2^{-/-} or MyD88^{-/-} macrophages after LM infection indicating that PI3K is critically involved in the downstream signaling pathway during phagocytosis of LM by WT macrophages.

1 The Rho GTPases, Rac1 and Cdc42, are widely known as the critical regulators of actin
2 cytoskeletal rearrangements during the phagocytic response to a variety of extracellular
3 stimuli (10, 12). It has been reported that PI3K signaling is involved in the activation of
4 the Rho family GTPases, including Rac1 and Cdc42, leading to the initiation of actin
5 polymerization (21). In our study, we found that inhibition of the Rho family GTPases
6 by toxin B reduced phagocytosis of LM and that activation of Rac1, but not Cdc42, was
7 far less significant in TLR2^{-/-} and MyD88^{-/-} macrophages. In addition, the inhibitors for
8 PI3k and Rac1 never affected the expression of TLR2 on macrophages. Therefore it is
9 likely that TLR2-MyD88-mediates an enhancement of phagocytosis through PI3K and
10 Rac1 activation in WT macrophages after infection with LM. This is consistent with the
11 recent report by Shin *et al.* showing that downstream signaling of MyD88-mediated
12 phagocytosis is dependent on PI3K and Rac1 activation during *Borrelia* infection (48).
13 However, one recent study showed that a MyD88-independent activation of the actin-
14 Cdc42/Rac pathway is required for TLR mediated phagocytosis (30). In fact the authors
15 determined the phagocytosis of GFP-*E. coli* after LPS treatment, which is different from
16 our experiment that directly focuses on the role of TLR2 on early infection (after 1 h)
17 without treatment. Furthermore, Braun *et al.* (8) reported that LM induces activation of
18 PI3K to infect Caco-2 cells via InlB protein. Therefore, activation of PI3K appears to be
19 important for the internalization of LM irrespective of the type of host cell. Small Rho
20 GTPases are upstream activators of the Arp2/3 complex in many actin polymerization
21 events such as cell-cell adhesion, cell movement and phagocytosis (28). Moreover,
22 Sandra *et al.* (44) reported that Rac1 promotes recruitment of cortactin and activation of
23 Arp2/3 in E-cadherin mediated *Listeria* entry. A possibility that Arp2/3 might be the

1 downstream molecule for phagocytosis of LM by macrophages is to be determined in a
2 further study.

3
4 Although our results clearly indicate that TLR2-MyD88-dependent signals leading to
5 PI3K and Rac1 activation enhance the phagocytosis of LM by macrophages, the exact
6 interaction between TLR2 and its ligand for the induction of the MyD88-PI3K-Rac1
7 pathway still remains to be determined. Recent studies highlighted the immunological
8 function of lipoproteins derived from Gram-positive bacteria and lipoproteins have been
9 shown to induce the host immune response via activation of the TLR2 signaling
10 pathway. Machata *et al.* (40) have shown that surface lipoproteins derived from LM
11 activate the TLR2 signaling pathway during the early phase of infection. It is possible
12 that the interaction of TLR2 with lipoprotein expressed on the surface of LM is
13 involved in the TLR2-dependent enhancement of phagocytosis that was observed in our
14 study. On the other hand, little is known about receptors that contribute to the
15 phagocytosis of LM. It seems that one or more receptors, which are constitutively
16 expressed on macrophages including type I and type II scavenger receptors, may serve
17 as phagocytic receptors for LM (26). In this study, we found that CHX never affected
18 TLR2-dependent phagocytosis. This result suggests that enhancement of the
19 phagocytosis is not attributable to the newly synthesized phagocytic receptors after LM
20 infection. The exact manner of direct interaction between macrophages and LM remains
21 to be elucidated.

22
23 There is increasing evidence suggesting the involvement of TLR signaling in
24 phagocytosis of bacterial pathogens *in vitro* (41). However, the functional significance

of these finding *in vivo* has yet to be determined in many cases. In the present study, we showed that macrophages from TLR2^{-/-} and MyD88^{-/-} mice harbor significantly lower numbers of phagocytosed LM after i.p. and i.v. infection with LM compared with macrophages from WT mice in the early period of LM infection. This finding clearly indicates that the TLR2-MyD88-mediated enhancement of phagocytosis is not a phenomenon observed only under specific experimental conditions *in vitro* but is also applicable to *in vivo* infection.

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- 4

Figure Legends

Figure 1. Difference in the phagocytosis of *Listeria monocytogenes* (LM) between WT and TLR2^{-/-} macrophages. (A) WT macrophages were incubated with 40 µg ml⁻¹ anti-TLR2 mAb (IgG1) or mouse control IgG for 1 h, and infected with LM at a MOI of 20 for 1 h. Cells were then treated with 20 µg ml⁻¹ gentamicin for 30 min and the number of phagocytosed bacteria was enumerated. (B) WT, TLR2^{-/-} and TLR4^{-/-} macrophages were infected with LM at a MOI of 20 for 1 h. The number of phagocytosed bacteria was determined after treatment with gentamicin for 30 min. (C, D) WT and TLR2^{-/-} macrophages were infected with LM as mentioned above and the number of associating-LM (C) and the mean phagocytic index (D) were determined. The mean phagocyte index was calculated as follows: (the number of phagocytosed LM/the number of associating LM) × 100. (E) Bone marrow-derived macrophages from WT and TLR2^{-/-} macrophages were infected with LM at a MOI of 20 for 1 h and the number of phagocytosed bacteria was determined. (F) WT and TLR2^{-/-} macrophages were cultured with polystyrene beads for 2 h at a bead to cell ratio of 50 to 1. After washing, cells were fixed with 1% paraformaldehyde and the number of internalized beads was counted under fluorescence microscope. Data are expressed as the mean ± SD for triplicate cultures. Similar results were obtained in three independent experiments. * P < 0.05.

Figure 2. Fluorescence microscopic analysis for the phagocytosis of LM. (A) WT and TLR2^{-/-} macrophages were cultured with CFSE-labeled LM at a MOI of 50 for 1 h. After washing, cells were fixed and incubated with rabbit anti-*Listeria* Ab and anti-

rabbit IgG Ab conjugated with Alexa Fluor 594 to stain adherent LM to macrophages. According to the procedure, phagocytosed LM (green spots) was distinguished from adherent LM (yellow spots) in merged fluorescence images. (B, C) After staining infected LM with rabbit anti-*Listeria* Ab and Alexa Fluor 594-conjugated anti-rabbit IgG Ab, the number of associating LM and adherent LM was counted in 500 WT and TLR2^{-/-} macrophages, and the number of phagocytosed LM was determined. The phagocytic index was calculated as follows: phagocytic index (%) = (the number of phagocytosed LM/the number of associating LM) × 100. The average number of associating LM (B) and mean phagocyte index (C) are depicted. * P < 0.05. (D) Columns depict the exact distribution of the phagocytic index in WT and TLR2^{-/-} macrophages. The experiment was repeated three times and data are expressed as the mean ± standard error for three independent experiments. (E) WT and TLR2^{-/-} macrophages were infected with CFSE-labeled LM at a MOI of 50 for 1 h and incubated with CTC for 15 min. Cells were fixed and treated with rabbit anti-*Listeria* Ab and Alexa Fluor 350-conjugated anti-rabbit IgG Ab. The numbers of viable and dead bacteria inside macrophages were counted. The viability of intracellular bacteria (%) was calculated as follows: The number of viable LM/(the number of viable and dead LM) × 100.

Figure 3. Difference in the phagocytosis of LM between WT and MyD88^{-/-} macrophages. (A) WT and MyD88^{-/-} macrophages were infected with LM at a MOI of 20 for 1 h. After washing, cells were treated with gentamicin for 30 min and the number of phagocytosed bacteria was determined. Data are expressed as the mean ± SD for triplicate cultures. Similar results were obtained in three independent experiments. * P <

0.05. (B) WT and MyD88^{-/-} macrophages were infected with CFSE-labeled LM at a MOI of 50 for 1 h. The number of phagocytosed LM and adherent LM was evaluated after immunostaining. Data are expressed as the means \pm SD for triplicate cultures. Similar results were obtained in two independent experiments. * P < 0.05. (C) WT and MyD88^{-/-} macrophages were incubated with polystyrene beads for 2 h. After washing, cells were fixed and the number of internalized beads was counted. (D) WT and MyD88^{-/-} macrophages were infected with CFSE-labeled LM at a MOI of 50 for 1 h and treated similarly to Fig. 2E. The viability of intracellular bacteria (%) was calculated. (E) WT, TLR2^{-/-} and MyD88^{-/-} macrophages were stained with FITC-conjugated anti-TLR2 mAb (IgG2b) or control IgG2b, and the level of TLR2 expression was analyzed by FACS. The histogram with the bold line shows the level of TLR2 expression on the three types of macrophages. The hatched area represents the basal fluorescent intensity in cells treated with control Ab. (F) Whole PEC and adherent PEC obtained from WT (red), TLR2^{-/-} (blue) and MyD88^{-/-} (green) mice were stained with PE-conjugated anti-F4/80 mAb (IgG2a). The expression of F4/80 was analyzed by FACS. The hatched area represents the basal fluorescent intensity in cells treated with control IgG2a.

Figure 4. The effect of LPS and cycloheximide on the phagocytosis of LM by TLR2^{-/-} and MyD88^{-/-} macrophages. (A) WT and TLR2^{-/-} macrophages were infected with LM at a MOI of 20 in the presence or absence of 100 ng ml⁻¹ Pam3CSK4 or 100 ng ml⁻¹ LPS for 1 h, and the number of phagocytosed LM was determined. Data are expressed as the mean \pm SD for triplicate cultures. * P < 0.05. (B) WT, TLR2^{-/-} and MyD88^{-/-} macrophages were infected with LM at a MOI of 20 in the presence or absence of 100 ng ml⁻¹ LPS for 1 h, and the number of phagocytosed LM was counted.

Data are expressed as the mean \pm SD for triplicate cultures. * $P < 0.05$. (C) WT and TLR2^{-/-} macrophages were infected with LM at a MOI of 20 for 1 h in the presence or absence of CHX, and the number of phagocytosed bacteria was determined. Data are expressed as the mean \pm SD for triplicate cultures. Similar results were obtained in three independent experiments.

Figure 5. The effect of LY294002 on the phagocytosis of LM and difference in the phosphorylation of Akt between WT, TLR2^{-/-} and MyD88^{-/-} macrophages after LM infection. (A) WT macrophages were infected with LM at a MOI of 20 in the presence of graded concentrations of LY294002, a PI3K inhibitor, and the number of phagocytosed bacteria was determined. Data are expressed as the mean \pm SD for triplicate cultures. (B) WT, TLR2^{-/-} and MyD88^{-/-} macrophages were infected with LM at a MOI of 20 for 1 h in the presence or absence of 20 μ M LY294002, and the number of phagocytosed bacteria was determined. Data are expressed as the mean \pm SD for triplicate cultures. Similar results were obtained in two independent experiments. * $P < 0.05$. (C) WT, TLR2^{-/-} and MyD88^{-/-} macrophages were infected with LM at a MOI of 20 for 15 and 30 min. Cells were lysed and phosphorylated Akt was evaluated by Western blotting. Total Akt was used as a loading control.

Figure 6. The effect of toxin B on the phagocytosis of LM and difference in the phosphorylation of Rho family GTPase proteins between WT, TLR2^{-/-} and MyD88^{-/-} macrophages after LM infection. (A) WT, TLR2^{-/-} and MyD88^{-/-} macrophages were infected with LM at a MOI of 20 for 1 h in the presence or absence of 40 ng ml⁻¹ toxin B, and the number of phagocytosed bacteria was determined. Data

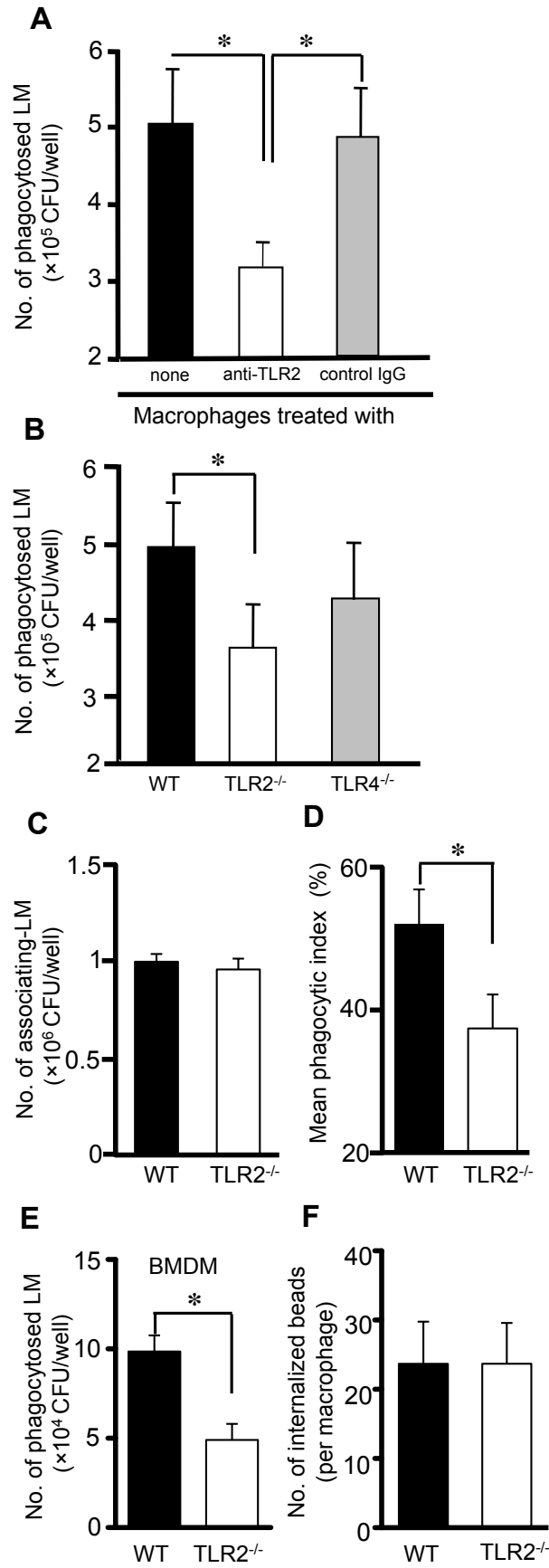
are expressed as the mean \pm SD for triplicate cultures. Similar results were obtained in two independent experiments. * $P < 0.05$. (B) WT, TLR2^{-/-} and MyD88^{-/-} macrophages were infected with LM at a MOI of 20 for 15 and 30 min. The GTP-bound forms of Rac1 and Cdc42 were precipitated using glutathione *S*-transferase (GST)-bound p21-activated kinase 1 (PAK1) and quantified by Western blotting using anti-Cdc42 (IgG1) and anti-Rac1 (IgG2b) mAbs. Total Rac1 and Cdc42 in the cell lysate were used as a loading control.

Figure 7. The effect of LY294002 and toxin B on the expression of TLR2 on macrophages. WT, TLR2^{-/-} and MyD88^{-/-} macrophages were incubated with 20 μ M LY294002 or 40 ng ml⁻¹ toxin B for 1 h and treated with anti-CD16/32 mAb (IgG2b), followed by staining with FITC-conjugated anti-TLR2 mAb (IgG2b) or control IgG2b. The level of TLR2 expression was analyzed by FACS. The histogram with the red line shows the level of TLR2 expression on macrophages without treatment, blue line represents the level of TLR2 expression on macrophages treated with LY294002, and green line shows the level of TLR2 expression on macrophages treated with toxin B. The hatched area represents the basal fluorescent intensity in cells treated with control IgG2b.

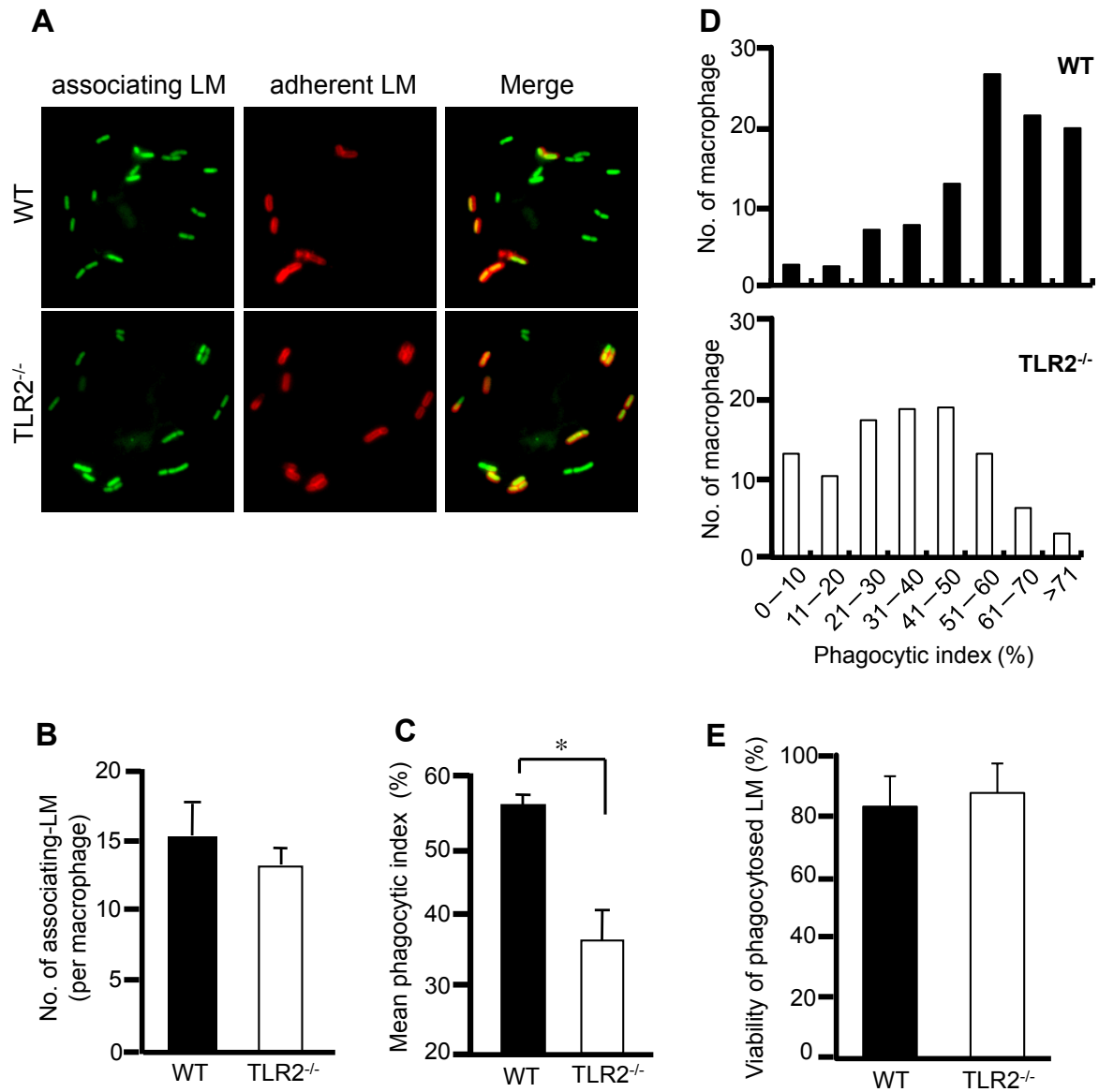
Figure 8. Difference in the phagocytosis of LM and bacteria burden between WT and TLR2^{-/-} mice after LM infection *in vivo*. (A) WT, TLR2^{-/-} and MyD88^{-/-} mice were injected i. p. with 3 ml of 3% thioglycollate medium and infected i. p. with 1×10^8 CFU of LM 4 days later. Five min after infection, PECs were collected and the number of phagocytosed LM was determined. * $P < 0.05$. (B) WT and TLR2^{-/-} mice were

- 1 infected intravenously with 10^4 CFU of LM. Spleen was removed 1 day after infection
- 2 and the number of viable bacteria in spleen was determined. * $P < 0.05$.
- 3

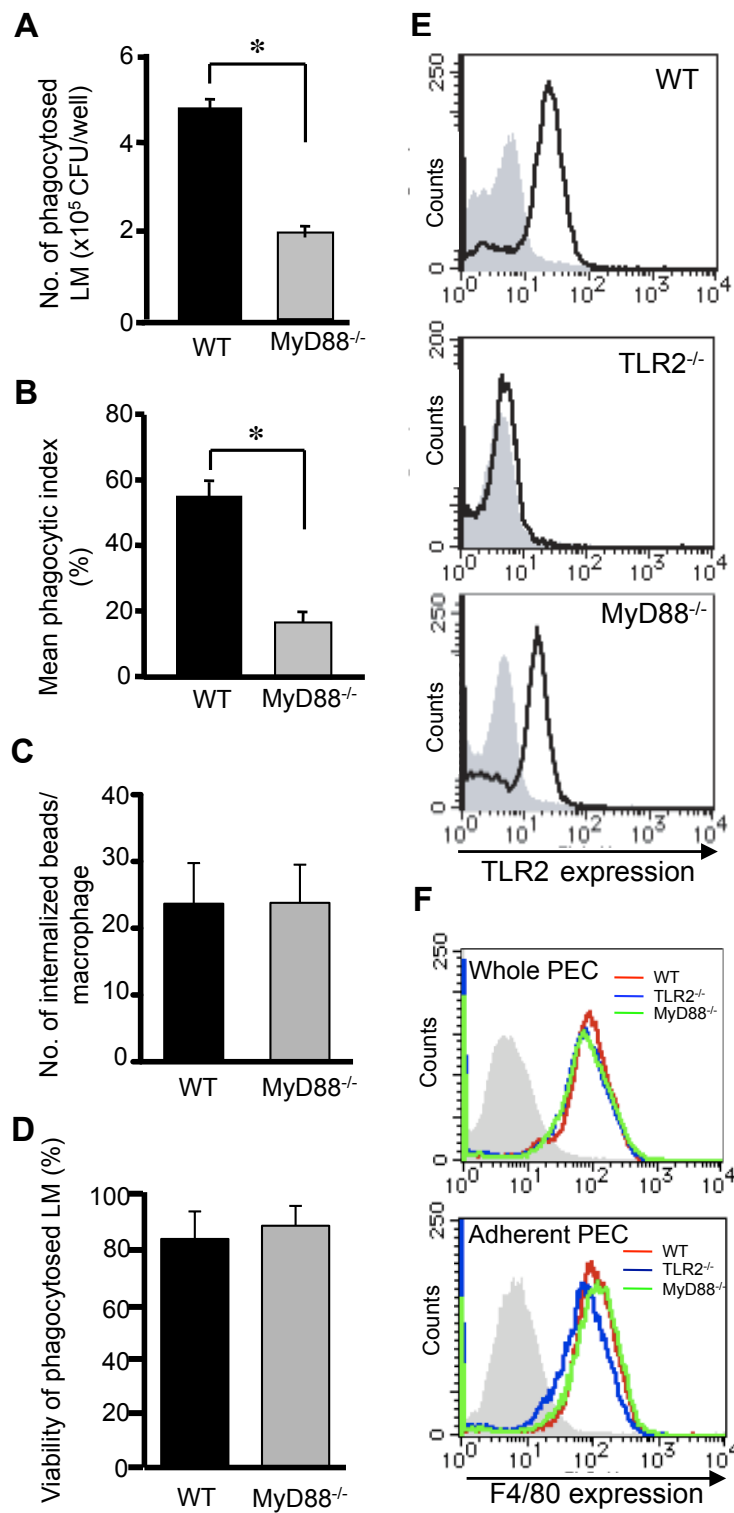
Shen et al.
Fig. 1



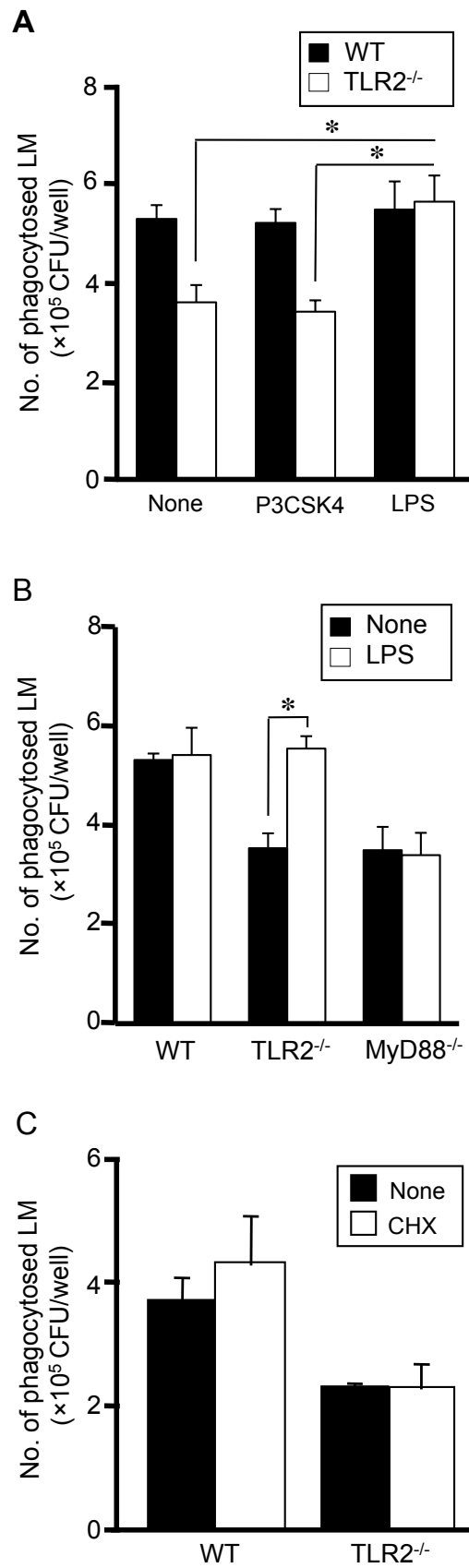
Shen et al.
Fig. 2



Shen et al.
Fig. 3



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Fig. 4



A

No. of phagocytosed LM ($\times 10^5$ CFU/well)

LY294002 (μ M)

LY294002 (μ M)	No. of phagocytosed LM ($\times 10^5$ CFU/well)
-	~6.5
5	~5.3
10	~4.1
20	~3.4
40	~3.4

B

No. of phagocytosed LM ($\times 10^5$ CFU/well)

Legend: ■ None, □ LY294002

Genotype	None	LY294002
WT	~5.8	~2.5
TLR2 ^{-/-}	~3.8	~3.0
MyD88 ^{-/-}	~3.1	~2.7

C

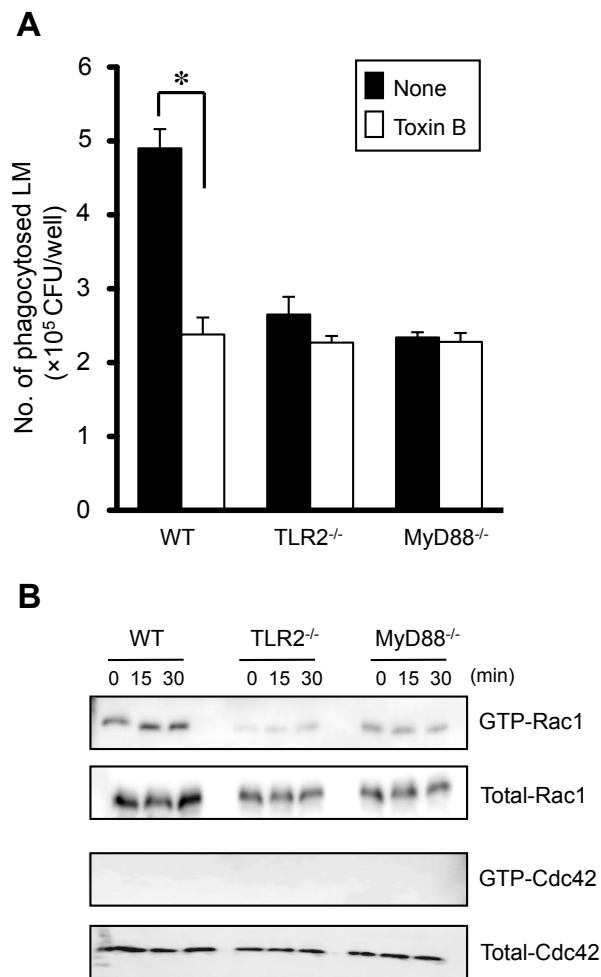
WT TLR2^{-/-} MyD88^{-/-}

0 15 30 0 15 30 0 15 30 (min)

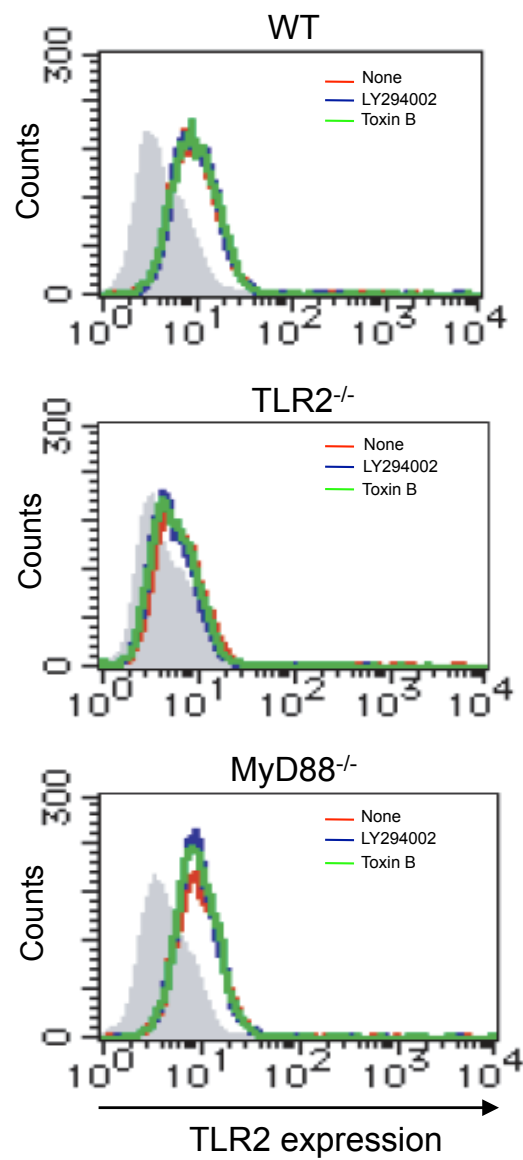
pAkt

Akt

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Fig. 6



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Fig. 7



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Fig. 8

